JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2006, p. 3086–3093 Vol. 44, No. 9
0095-1137/06/$08.00+0 doi:10.1128/JCM.02672-05

Enhanced Patient Serum Immunoreactivity to Recombinant *Mycobacterium tuberculosis* CFP32 Produced in the Yeast *Pichia pastoris* Compared to *Escherichia coli* and Its Potential for Serodiagnosis of Tuberculosis

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Received 23 December 2005/Returned for modification 13 February 2006/Accepted 11 June 2006

CFP32 is a *Mycobacterium tuberculosis* complex-restricted secreted protein that was previously reported to be present in a majority of sputum samples from patients with active tuberculosis (TB) and to stimulate serum antibody production. CFP32 (originally annotated as Rv0577 and also known as TB27.3) was therefore considered a good candidate target antigen for the rapid serodiagnosis of TB. However, the maximal sensitivity of CFP32 serorecognition may have been limited in earlier studies because recombinant CFP32 (rCFP32) produced in *Escherichia coli* was used as the test antibody-capture antigen, a potential shortcoming stemming from differences in bacterial protein posttranslational modifications. To further investigate the serodiagnostic potential of rCFP32 synthesized in different heterologous hosts, we expressed rCFP32 in the yeast *Pichia pastoris*. Compared to *E. coli* rCFP32, yeast rCFP32 showed a higher capacity to capture polyclonal antisera in Western blot studies. Likewise, yeast rCFP32 was significantly better recognized by the sera from TB patients and healthy *Mycobacterium bovis* bacillus Calmette-Guérin (BCG)-vaccinated individuals, by enzyme-linked immunosorbent assay (ELISA), than *E. coli* rCFP32. In subsequent testing, the yeast rCFP32-based antibody-capture ELISA had a sensitivity of 85% and a specificity of 98% for the discrimination of active TB cases ($n = 40$) from BCG vaccinees ($n = 39$). The sensitivity was surprisingly high for a single-antigen TB serodiagnostic test compared to tests using *E. coli*-expressed antigens. Overall, the *trans*-production of rCFP32 in *P. pastoris* significantly improved the serologic detection of CFP32-specific antibodies in patient sera, thereby offering a new, possibly better, modality for producing antigens of diagnostic potential for use in the development of immunoassays for both TB and other infectious diseases.

Over one-third of the world's population is infected with *Mycobacterium tuberculosis*, the etiologic agent of tuberculosis (TB) (10). Annually, this infection causes an estimated 8 million new TB cases and 3 million deaths. Rapid diagnosis and case identification, to minimize transmission to susceptible individuals, is critical in the control of TB. Current methods either have a low sensitivity, are slow to definitive results, or require methods and infrastructure not available in most resource-poor countries burdened by the majority of TB and human immunodeficiency virus cases. In most developing countries, less than 50 to 60% of patients are diagnosed by the microscopic identification of acid-fast bacilli (AFB) in sputum smears (29). The remaining are eventually diagnosed by clinical criteria alone. Therefore, the availability of a sensitive, affordable, and practical point-of-care diagnostic test for rapid TB case identification and treatment would be of substantial benefit. As with current standard-of-care TB diagnostic tests, the sensitivities and specificities of most currently available serodiagnostic tests for TB need significant improvement (21). One major difficulty is that the spectrum of *M. tuberculosis* antigens recognized by sera varies dramatically between patients. Depending upon the antigen, its method of production (as a purified native *M. tuberculosis* protein versus a recombinant), and its use in combination with other antigens, the sera of 12 to 96% of TB patients have been found to contain specific antibodies to the test antigens (16, 17, 30, 36). However, even with the same method of manufacture, the serum recognition of even the most promising TB antigens can vary widely depending upon the country of origin of the studied cohort as well as the AFB smear status and disease manifestations of the individuals within the population (17, 26, 36). Therefore, the evaluation of additional potential candidate antigens for serodiagnosis is necessary to maximize TB test performance across cohorts.

As a contribution to this effort, we identified and investigated a secreted *M. tuberculosis* protein named CFP32 (also known as TB27.3 and originally called Rv0577) (5, 7) that is

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unique to tubercle bacilli, is expressed during infection, and is 1 of only approximately 30 known serum-reactive *M. tuberculosis*-secreted or released antigens (13, 14). Interestingly, *M. tuberculosis* clinical isolates appeared to express more CFP32 than laboratory-adapted *M. tuberculosis* strains and other *M. tuberculosis* complex (MTC) subspecies (including *Mycobacterium bovis* bacillus Calmette-Guérin [BCG]) in a limited evaluation (13). We have also reported on the development of specific CFP32 serum antibody-capture enzyme-linked immunosorbent assays (ELISAs) for TB diagnosis (two variants were described). These "first-generation" anti-CFP32 antibody-capture immunoassays using *Escherichia coli* recombinant protein were able to detect a statistically significant specific serological response in 30 to 34% of known TB patients from Brazil (12 of 35) and India (9 of 30). As such, these data approached the sensitivity of AFB smear for TB diagnosis. Importantly, unlike AFB smear, cavitary TB status did not increase the likelihood of having a positive anti-CFP32 serologic response, and the specificity was as high as 98% (1 of 40 control non-TB persons was positive). Since the majority of non-TB control donors were tuberculin skin test (TST) positive (reactive to a subcutaneous placement of *M. tuberculosis* purified protein derivative [PPD]) for *M. tuberculosis* exposure, the data indicated that the high antibody recognition of CFP32 is associated with clinical disease (13). As such, CFP32 is a very promising candidate for inclusion in a multiantigen TB serodiagnostic test.

However, with regard to the preceding work (14), the anti-CFP32 antibody-capture assay was developed using recombinant CFP32 (rCFP32) produced by *E. coli*, a recognized potential limitation (27). Differential protein processing, folding, and posttranslational modifications (e.g., glycosylation, phosphorylation, and acylation) exist in some *M. tuberculosis*-expressed proteins compared to the *E. coli*-expressed recombinant versions (22, 23) and can be of functional consequence (39). Several studies have reported that antibodies and T cells from TB patients that reacted to native *M. tuberculosis* proteins failed to react to the same protein produced by *E. coli* (22, 23, 25, 27). These findings underscore the importance of posttranslational modifications in adding antigenic epitopes. Indeed, the removal of glycosyl residues from mycobacterial protein(s) has been shown to reduce host immune responses to the "naked" antigen (11, 23). Analysis of the CFP32 protein indicates the presence of several potential posttranslational modification sites (3a). Therefore, due to our CFP32 immunoassay's dependence on a potentially suboptimal test antigen, the 30 to 34% sensitivity of the first-generation anti-CFP32 serodiagnostic ELISA may not reflect the actual degree of CFP32 antigenicity nor represent the true potential that an anti-CFP32 antibody-capture assay could achieve with a source of rCFP32 that better approximates native CFP32.

To address this issue, we chose to express CFP32 using the yeast *Pichia pastoris* as an alternative surrogate host to *E. coli* (3a). *P. pastoris* is a commonly employed laboratory and commercial organism known for its ability to produce high amounts of recombinant proteins in intracellular and secreted form (8, 18, 24, 28, 33). *Pichia pastoris* also has the ability to efficiently produce recombinant proteins that retain their functional properties (18, 20, 37). As with *M. tuberculosis*, *P. pastoris* has a codon usage that is GC rich (28). Therefore, it was thought that yeast may be a preferable host for the production of functional mycobacterial recombinant proteins and that protein posttranslational events in yeast may better mimic those of mycobacteria, resulting in a more seroreactive rCFP32.

In this study, the immunoreactivity of yeast-produced rCFP32 was compared to that of *E. coli*-produced rCFP32 by Western blotting and by using a serum antibody-capture ELISA that incorporated one or the other rCFP32 as the test antigen. Combined, the data indicated that rCFP32 produced in yeast was more immunogenic than rCFP32 produced in *E. coli*. The TB diagnostic potential of the yeast rCFP32 immunoassay was then evaluated using the sera from pulmonary TB patients and controls and was found to be highly sensitive and specific for the serologic diagnosis of TB. Our data highlight the importance of the source of recombinant antigen in the development of a high-performance TB serodiagnostic test.

MATERIALS AND METHODS

Expression of CFP32 in *P. pastoris*. rCFP32 was produced in the yeast *P. pastoris* as recently described (Benabdesselem et al., submitted). Briefly, *cfp32* (*rv0577*) cDNA was expressed in *Pichia pastoris* strain KM71H as a fusion c-Myc epitope, six-histidine (His_6)-tagged recombinant protein using the plasmid pPICZα under the control of the strong AOX1 promoter (Invitrogen Corporation, Carlsbad, Calif.). This construct also contained a *Saccharomyces cerevisiae* α mating factor prepropeptide secretion signal that is cleaved during protein processing. rCFP32 was purified using an Ni^+-Sepharose Fast Flow column (Amersham, Piscataway, N.J.) and dialyzed against phosphate-buffered saline (PBS). As a control for the potential effects due to gene *trans*-expression and antibiotic pressure, a second near identical plasmid was created but with the *cfp32* cassette out of frame, resulting in a failure to produce recombinant protein (data not shown).

Western blot analysis of native *M. tuberculosis* CFP32, yeast-produced rCFP32, and rCFP32 generated in *E. coli*. The preparation of *M. tuberculosis* culture filtrate, expression and purification of *E. coli* rCFP32, and the generation of anti-*E. coli* rCFP32 in rabbit were previously described (13). Murine antiserum to yeast-produced rCFP32 was also generated using a classical immunization protocol. Protein assays and Western blot analysis were performed as previously reported (13). Briefly, following electrophoresis of native CFP32 (present in *M. tuberculosis* culture filtrate), as well as yeast and *E. coli* rCFP32 proteins, and transfer to nitrocellulose, the membrane was reacted with rabbit anti-*E. coli* rCFP32 sera (1:3,000 dilution in blocking buffer), mouse anti-yeast rCFP32 sera (1:2,000 dilution), or anti-His_6-tagged monoclonal antibody (MAb) (2 μg in 5 ml of blocking buffer; QIAGEN, Valencia, Calif.), and then reacted with the appropriate MAb-linked horseradish peroxidase (Amersham, Piscataway, N.J.). Images were developed using ECL Western blot detection reagents (Amersham) and then exposed to Kodak BioMax film. Protein quantification of *M. tuberculosis* lysate, as well as yeast and *E. coli* rCFP32, was performed using the Bio-Rad protein assay (Bio-Rad, Hercules, Calif.). This protein quantification was repeated several times over, by more than one individual, and in side-by-side comparisons on some occasions. The variance in protein content between evaluations was never more than ~15%. Western blot data are illustrated in Fig. 1.

Study population. Tunisia is a country in Northern Africa with a TB case rate of 19.6/100,000 persons. In the preliminary immunoassay evaluation (Fig. 2), the sera from TB patients ($n = 25$) were randomly selected from a repository of patient sera collected during the initial clinical and biological testing for TB. Active pulmonary TB was confirmed by clinical, radiological, and bacteriological investigation. Sera from healthy subjects ($n = 17$) were randomly selected from a repository of sera remaining from clinical testing of student and/or staff members at the Pasteur Institute, Tunis. In the follow-up immunoassay evaluation (Fig. 3), additional serum samples were randomly selected from the same repository as used previously. Demographics for TB patients ($n = 40$) are as follows: 33 males and 7 females; mean age, 31 years; age range, 17 to 44 years. Demographics for healthy subjects ($n = 39$) are as follows: 23 males and 16 females; mean age, 28 years; age range, 22 to 54 years. Two additional healthy subjects, initially investigated, were excluded from the analysis because one person was revaccinated a third time upon entry into the university at the age of 20 and the second person had a 15-mm induration in response to the tuberculin skin test,

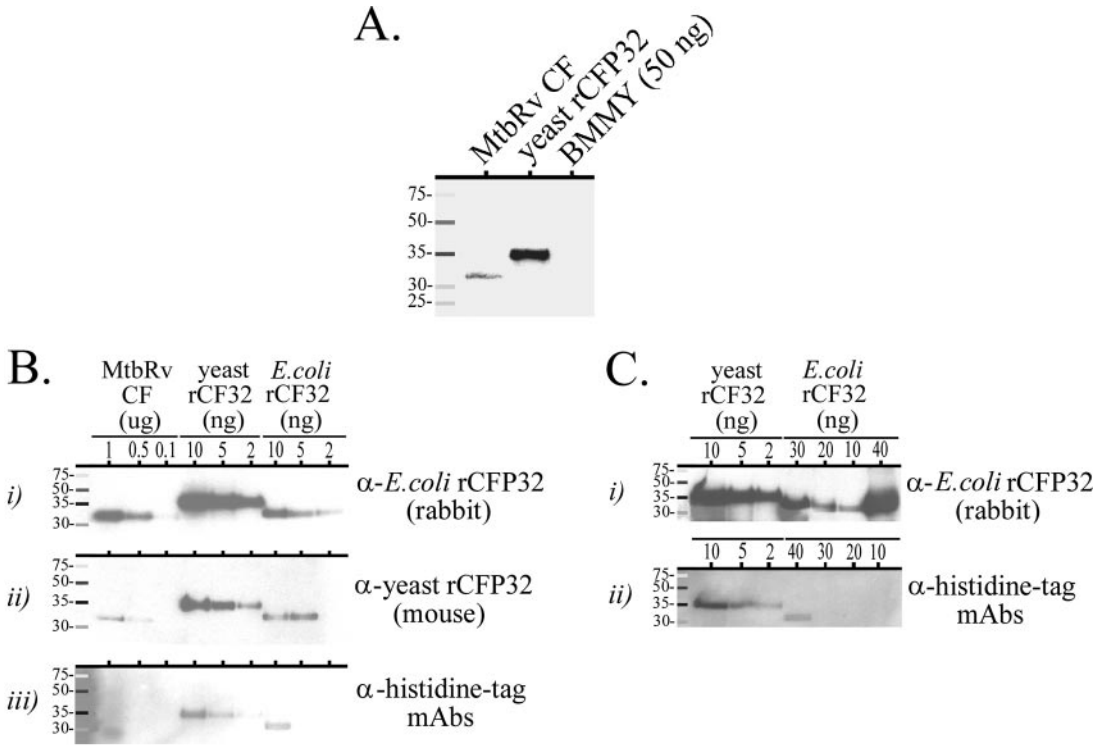

FIG. 1. Comparative study of antibody recognition of native *M. tuberculosis* CFP32 and rCFP32 expressed in *P. pastoris* and *E. coli*. (A) *trans*-expression of the CFP32 gene in yeast as detected by Western blotting. The detection of rCFP32 produced by pPICZ.*cfp32*-transformed yeast was done using rabbit anti-*E. coli* rCFP32 as the primary antiserum, 1 μg of *M. tuberculosis* H37Rv culture filtrate (MtbRv CF), 10 ng of His_6-tagged purified yeast rCFP32, and a volume (equal to 50 ng total protein) of concentrated filtered yeast culture medium in which yeast transformed with the missense CFP32 gene expression vector were grown (BMMY). (B) Western blot analyses point to a relative preferential antibody avidity for yeast-expressed rCFP32 compared to *E. coli*-expressed rCFP32. Native CFP32 in *M. tuberculosis* H37Rv CF and yeast- or *E. coli*-produced rCFP32, at the indicated input protein quantities, were detected by Western blot using anti (α)-*E. coli* rCFP32 (rabbit) (*i*) or anti-yeast rCFP32 (mouse) (*ii*) antisera or anti-His_6-tagged MAb (*iii*). The figure data are representative of results from three separate experiments; in one experiment (not shown), a membrane was successively stripped and reprobed with an alternative antiserum. (C) rCFP32 produced in yeast is relatively more antibody reactive than rCFP32 *trans*-expressed in *E. coli*. Western blotting was performed using various indicated input amounts of rCFP32 produced in yeast or *E. coli* as detected by anti-*E. coli* rCFP32 (rabbit) antisera (*i*) and anti-His_6-tagged MAb (*ii*). The figure is derived from two different blots probed with either anti-*E. coli* rCFP32 (rabbit) antisera or anti-His_6-tagged MAb. For each of the above blots, band sizes of molecular mass markers (kDa) are indicated on the left in the panel.

indicating recent exposure to *M. tuberculosis* (35). Chest X ray and sputum culture were negative in the latter case, but extrapulmonary disease was not definitively excluded. Otherwise, the PPD status of the healthy BCG-vaccinated controls was not known. BCG vaccination is nearly universal in Tunisia and given first at birth and then a second time upon entry into primary school. Only persons with suspected or with known immunodeficiency are not given BCG. Stored sera from 4 infants who were *M. tuberculosis* uninfected (PPD negative) and BCG vaccine naive (each infant was born to a family known to have had a child with a primary immunodeficiency and was later proven to be immunocompetent) were used as negative controls. All of the serum specimens were from Tunisian individuals with no human immunodeficiency virus infection.

Detection of human anti-CFP32 antibodies by ELISA. Polystyrene 96-well plates were coated with 100 μl of 1 μg/ml *E. coli* or *P. pastoris* rCFP32 in 0.05 M carbonate buffer (pH 9.6), incubated overnight at 4°C, washed once with PBS (pH 7.2) plus 0.05% Tween 20 (Sigma, St. Louis, Mo.) (PBS-T), blocked with 1.5% milk in PBS-T at room temperature (RT) for 1 h, and then washed three times with PBS-T. Serum samples diluted 1:200 in PBS were then added, incubated for 2 h at RT, and washed with PBS-T. To determine the amount of antibodies bound, wells were incubated for 1 h with alkaline phosphatase-linked sheep anti-human immunoglobulin (Amersham) diluted 1:3,000 in PBS-T, washed with PBS-T, developed with the alkaline phosphatase substrate *O*-phenylenediamine–H_2O_2 (Sigma) for 20 min of incubation at RT, and then the reaction was stopped with 100 μl of 4 N sulfuric acid. The enzymatic conversion of the substrate was quantified in a Multiskan.EX ELISA reader (Labsystems, Helsinki, Finland) at 492 nm as optical density (OD) units.

Data analysis. The ELISA results from TB patients were analyzed using a cutoff value equal to the mean OD for the serum samples from the healthy BCG-vaccinated controls plus 2 standard deviations (SD). For statistical analyses of the data, the differences between groups of TB patients and healthy BCG-vaccinated controls or PPD-negative–BCG-naive healthy controls were compared using the paired *t* test, unpaired *t* test, or Fisher's exact test, as indicated. Differences were considered statistically significant if the *P* value was <0.05.

RESULTS

Differential antibody recognition of native and recombinant forms of CFP32 antigen. We recently reported on the cloning and expression of *M. tuberculosis* CFP32 in *P. pastoris* (3a). To our knowledge, this was the first report on the immunoreactivity of an *M. tuberculosis* antigen produced in yeast. To reconfirm the correct cloning of CFP32 in *P. pastoris*, Western blot analysis was performed upon the culture filtrate (CF) of *M. tuberculosis* strain H37Rv (previously determined to contain CFP32) (13), His_6-tagged purified yeast rCFP32, and a sample of cell-free BMMY yeast culture medium in which the *P. pastoris cfp32* missense cassette transformant had been grown. Antisera raised in rabbit against *E. coli*-produced rCFP32 recognized both the native CFP32 in the CF of *M. tuberculosis* strain H37Rv and the yeast-produced rCFP32 but not the yeast culture medium (BMMY) negative control (Fig. 1A). The

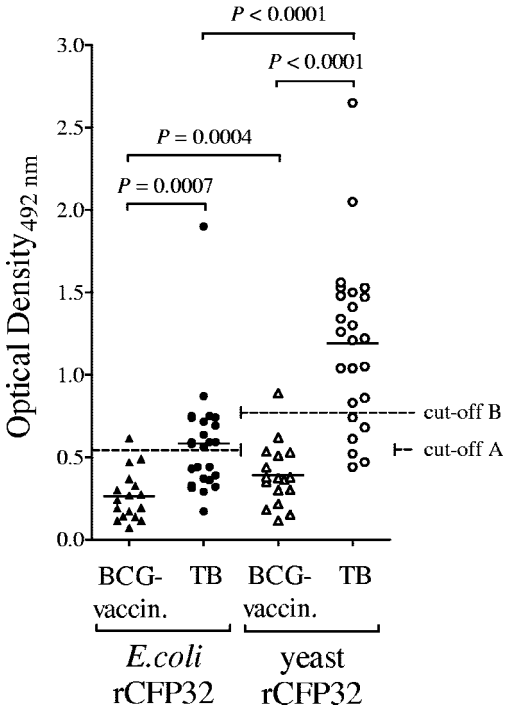

FIG. 2. Sera from human test subjects show greater reactivity to yeast-expressed rCFP32 than to *E. coli*-produced rCFP32. Using a home brew ELISA, the sera from 25 active TB case patients (TB), along with the sera of 17 BCG-vaccinated healthy persons (BCG-vaccin.), were cross-compared for immunoreactivity (measured in OD_{492} units) to two preparations of rCFP32 that were generated using either *E. coli* or *P. pastoris* as the surrogate host. The results of statistical analyses of the mean serological responses are shown (calculated using either the paired *t* test or unpaired *t* test, as indicated in the text). Each data point represents one patient. Solid horizontal lines represent the means of each category, while dashed horizontal lines represent the cutoff values above which serological responses to rCFP32 were deemed positive. Cutoff values were determined using the mean for the healthy BCG-vaccinated persons plus 2 standard deviations per the *E. coli* rCFP32 (cutoff A) or the yeast rCFP32 (cutoff B). OD_{492} values above the cutoff are taken to indicate the presence of serum antibodies to native CFP32 resulting from an immune response to an ongoing tubercle bacillus infection. As described in the text, statistical analyses of the data (Fisher's exact test) indicated that the rCFP32 produced in *P. pastoris* was superior to that *trans*-expressed in *E. coli* for the serodiagnosis of TB.

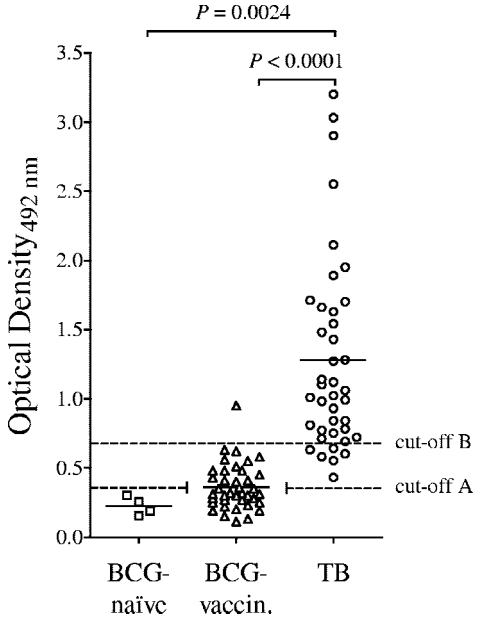

FIG. 3. Evaluation of a serological test for TB that incorporates rCFP32 *trans*-expressed in yeast. Using a home brew ELISA, the sera from 40 active TB case patients (TB), 39 BCG-vaccinated healthy persons (BCG-vaccin.), and 4 healthy PPD-negative–BCG-naive infants (BCG-naive) were compared for immunoreactivity (measured in OD_{492} units) to rCFP32 generated using *P. pastoris* as the surrogate host. Each data point represents one patient. Solid horizontal lines represent the means of each category, while dashed horizontal lines represent the cutoff values above which serological responses to rCFP32 were deemed positive and were determined using the mean for the healthy PPD-negative–BCG-naive infants (cutoff A) or the healthy BCG-vaccinated persons (cutoff B) plus 2 standard deviations. OD_{492} values above cutoff B are taken to indicate the minimal presence of serum antibodies to native CFP32 resulting from an immune response to an ongoing tubercle bacillus infection. The associated results of statistical analyses of the data using Fisher's exact test are shown. The results of statistical analyses of the mean serological responses (calculated using the unpaired *t* test) are provided in the text and supported a statistically significant difference in the means of each control group compared to the TB cohort.

band for native CFP32 ran at approximately 32 kDa, hence its name, while that of yeast rCFP32 appeared at 35 kDa. The higher band mass of the yeast rCFP32 is likely attributed to the c-Myc and His_6 tag (3a), but it may also have potentially received a contribution from *P. pastoris*-specific posttranslational modifications as well. The presence of the c-Myc epitope in the purified yeast rCFP32 product was previously confirmed by Western blot using a specific anti-Myc MAb (3a).

The immunogenicity of the CFP32 proteins from various sources was then comparatively evaluated in Western blot experiments using different specific reactive antibody preparations. These included the polyclonal rabbit antiserum raised against *E. coli*-expressed rCFP32, a polyclonal mouse antiserum raised against yeast-expressed rCFP32, and a commercially available anti-His_6 tag MAb of murine origin (Fig. 1B). Each antibody preparation reacted more strongly towards the yeast rCFP32 than the *E. coli*-expressed rCFP32 for the same calculated amount of input protein, regardless of the immunogen originally used to derive the antibodies or the respective species in which the antibodies were raised (Fig. 1B). A dilutional effect was also seen for each preparation, and consistent with previous observations, the *E. coli* rCFP32 ran at approximately 33 kDa. Furthermore, the native CFP32 was recognized by antisera raised to both recombinant proteins but not the anti-His_6 tag MAb, as was anticipated. These data also further demonstrate the presence of the His_6 tag in the purified yeast rCFP32 protein. In this regard, it should be noted that the His_6 tag was placed N-terminal in the *E. coli* rCFP32 but C-terminal in the yeast rCFP32. However, both preparations of purified rCFP32 were confirmed to possess predominantly full-length protein by silver and/or Coomassie blue staining (3a, 13). Therefore, it was contrary to expectations that the band intensities for each respective amount of input protein of yeast and *E. coli* rCFP32 were not equal in the blot probed with the anti-His_6 tag MAbs. Again, as described in Materials and Methods, much effort was dedicated to ensuring the accuracy of recombinant protein quantification, and the calculated amounts of input protein are believed to have been correct.

These data therefore suggested either a variance in the relative immunogenicity of the yeast and *E. coli* rCFP32 proteins or that perhaps the C-terminal position of the His_6 tag in yeast rCFP32 is better exposed and consequently might be better recognized than that of the *E. coli* form.

To qualitatively estimate the inequality in antibody avidity between the two rCFP32 preparations, the Western blot was repeated using a broader range of protein input amounts (Fig. 1C). As a result, using the anti-*E. coli* rCFP32 antiserum, the relative band intensity of 2 μg of yeast rCFP32 appeared similar to 30 μg of *E. coli*-expressed rCFP32, suggesting a 15-fold difference in immunoreactivity. The basis of this disparate capacity to capture antibodies remains to be determined but may stem from differences in the nature of posttranslational protein processing in *P. pastoris* and *E. coli*, which could better avail the yeast rCFP32 to antibody recognition.

Differences in serological reactivity to *E. coli* and yeast rCFP32 antigens. In a previous study, 30 to 34% of TB patients living in Brazil or India exhibited a serologic response to *E. coli*-generated rCFP32 (13). Given the above-mentioned factors that may limit the immunological activity of antigens synthesized in *E. coli* (23, 27), we were interested in comparing the serologic response towards rCFP32 produced, respectively, in yeast and in *E. coli*. Using a random sample collection of sera from TB patients ($n = 25$) and healthy BCG-vaccinated persons ($n = 17$), we performed side-by-side challenges in a home brew ELISA incorporating one or the other rCFP32 as the test antibody-capture antigen. As shown in Fig. 2, the average serological response (in OD_{492} units) to yeast rCFP32 was much greater than the response to *E. coli* rCFP32 from patients with TB ($P < 0.0001$, paired t test) as well as the healthy BCG-vaccinated persons ($P = 0.0004$, paired t test). In fact, the sera from every TB patient and healthy BCG vaccinee that was tested reacted more strongly to the yeast rCFP32 than the *E. coli* rCFP32. Interestingly, there was a greater difference in mean OD_{492} ($\Delta OD_{492\ nm}$) response to yeast rCFP32, in comparison to *E. coli* rCFP32, in TB patient cases than there was in BCG-vaccinated persons ($\Delta OD_{492\ nm} = 0.75$ versus 0.31, respectively).

Moreover, the mean response of TB patients to *E. coli* rCFP32 ($OD_{492} = 0.58$) was significantly greater than that of the sera from BCG-vaccinated healthy persons ($OD_{492} = 0.27$) ($P = 0.0007$, unpaired t test). Indeed, similar to our previous study (13), more TB patient sera recognized the *E. coli* rCFP32 than did the non-TB controls. In fact, using a cutoff value given by the mean OD_{492} reading of the BCG-vaccinated persons to *E. coli* rCFP32 + 2 SD, 56% of TB patients (14 of 25) and 6% of BCG-vaccinated persons (1 of 17) were positive for a serological response to *E. coli* rCFP32 ($P = 0.001$, Fisher's exact test). These data from a Tunisian population therefore support the earlier reported findings with respect to the immunogenicity of CFP32 in humans from different parts of the world, although the overall percentage of serological responders to *E. coli* rCFP32 was higher in the Tunisian TB cohort (56%) than the previously evaluated TB cohorts from Brazil and India (30 to 34%) (13). It should be noted here that there was one TB patient with a serological response to *E. coli* rCFP32 that was greater than that of any other subject to this antigen ($OD_{492} = 1.9$) and was exceeded in magnitude only by this same person's response to yeast rCFP32 ($OD_{492} = 2.65$) and one other's (Fig. 2). These data argue against the possibility that errors in recombinant protein quantification were acting as a limiting factor determining differences in antibody affinity in this experiment and support the Western blot observations illustrated in Fig. 1.

As with the rCFP32 produced in *E. coli*, the mean OD_{492} of TB patients to yeast rCFP32 was also greater than that of the sera from BCG-vaccinated healthy persons (1.19 versus 0.39, respectively; $P < 0.0001$, unpaired t test) (Fig. 2). Using a cutoff value given by the mean OD_{492} reading of the BCG-vaccinated persons to yeast rCFP32 + 2 SD, 76% of TB patients (19 of 25) and 6% of BCG-vaccinated persons (1 of 17) were positive for a serological response to yeast rCFP32 ($P < 0.0001$, Fisher's exact test). Indeed, by this measure, more TB patients exhibited a serological response to yeast rCFP32 than did to *E. coli* rCFP32 (19 versus 14, respectively). Therefore, in this preliminary examination, in terms of both the magnitude of responses and the degree of TB case differentiation, the immunoassay utilizing yeast rCFP32 as the antibody-capture antigen outperformed the similar ELISA utilizing rCFP32 produced in *E. coli* as a surrogate measure for the presence of antibodies to *M. tuberculosis* CFP32 and as a serodiagnostic test for TB.

Evaluation of the yeast rCFP32-based ELISA for serodiagnosis of TB. To validate the diagnostic potential of the ELISA utilizing rCFP32 expressed in yeast as a serological test for TB, additional sera were tested. The total collection included samples from 40 patients with confirmed pulmonary TB, 39 healthy BCG-vaccinated individuals, and 4 healthy infants who were PPD negative–BCG vaccine naive (Fig. 3). In addition to serum samples tested for the first time, this cohort included each of the samples previously evaluated (Fig. 2) to prove consistency of results. In comparison to the PPD-negative–BCG-naive controls, the average serological response to yeast rCFP32 (in OD_{492} units) was significantly higher in TB patients ($P < 0.0001$, unpaired t test). The mean OD_{492} values for TB patients and PPD-negative–BCG-naive controls were 1.28 and 0.23, respectively (with a mean ΔOD_{492} of 1.05). Using a cutoff value given by the mean OD_{492} reading of the PPD-negative–BCG-naive controls + 2 SD, 100% of TB patients (40 of 40) and 0% of the controls (0 of 4) were positive for a serological response to yeast rCFP32 ($P = 0.0024$, Fisher's exact test). Compared to the BCG-naive controls, the serological response of the BCG-vaccinated subjects to the yeast rCFP32 was also higher. This was not an unexpected finding given that, as an MTC subspecies, BCG is known to possess the *cfp32* gene and to express CFP32 in vitro (13, 14). Interestingly, one healthy subject excluded from this analysis was revaccinated for a third time with BCG in early adulthood prior to blood donation and also exhibited a strong serologic response to CFP32 ($OD_{492} = 1.4$). In addition, there was another BCG-vaccinated person who was noted for having an extremely high serological response to the yeast rCFP32 ($OD_{492} = 1.8$). The subsequent tuberculin skin testing of this subject showed a 15-mm induration that is highly suspicious for recent exposure to *M. tuberculosis*. As a result, this person was also excluded from the data set.

Most noteworthy overall, however, was that the average serological response to yeast rCFP32 was significantly higher in the TB patients than in the remaining BCG-vaccinated healthy persons ($P < 0.0001$, unpaired t test). The mean OD_{492} value

for the BCG-vaccinated subjects was 0.36 (with a mean ΔOD_{492} of 0.91 compared to TB patients). Using a cutoff value given by the mean OD_{492} reading of the BCG vaccinees + 2 SD, 85% of TB patients (34 of 40) and 2.5% of BCG-vaccinated persons (1 of 40) were positive for a serological response to yeast rCFP32 ($P < 0.0001$, Fisher's exact test). Therefore, even using the more stringent BCG-vaccinated persons' sera for a reference cutoff, yeast rCFP32 was able to diagnose 85% of TB patients and misclassified only one BCG-vaccinated healthy serum sample as being reactive (or positive) for *M. tuberculosis* infection, resulting in a specificity of 98%. This donor also gave a positive response to both recombinant antigens in the preliminary evaluation (Fig. 2). All TB patients previously determined to be serologically positive for anti-CFP32 antibodies, as shown in Fig. 2, were positive in the secondary evaluation illustrated in Fig. 3. The positive predictive (97%) and negative predictive (90%) values of the yeast rCFP32 ELISA were very good as well. Therefore, even though a single antigen was utilized in the assay, the sensitivity and specificity of the yeast rCFP32 TB test approached or surpassed the values of these measures from multiantigen serodiagnostic tests for TB (16, 17, 36).

DISCUSSION

TB is a leading cause of morbidity and mortality worldwide. Indeed, the World Health Organization recently declared a new TB emergency for Africa, where the incidence rate of infections has tripled in many countries since 1990 (38). The lack of a low-cost, easy to perform, rapid, sensitive, and specific TB diagnostic test impedes patient treatment and transmission control measures, especially in resource-poor countries. Standard bacteriologic culture is slow, the sensitivity of AFB morphological identification is suboptimal, and molecular methods for diagnosis of TB based on nucleic acid amplification remain out of reach of most high-TB-burdened countries due to cost and technical restrictions. On the other hand, a diagnostic method for TB that directly detects patient antibodies to *M. tuberculosis* components has the technological potential to overcome these limitations and may provide additional benefits as well. For instance, a low-cost, real-time, point-of-care serologic test could theoretically be developed into a simple dipstick format for serum and/or urinary antibody detection, thereby minimizing infectious material handling and laboratory infrastructure. Such a TB test may also be applied to situations where diagnosis can be problematic, as with extrapulmonary TB patients, children, the elderly, and immunocompromised individuals or other smear-negative culture-negative cases. However, as currently devised, the serodiagnostic methods for TB are not adequately sensitive, poorly specific, or both (15). In the present study, we investigated whether the means of antigen production may be impeding the development of an optimized serodiagnostic test for TB.

In the case of *M. tuberculosis*, its slow growth, coupled with safety concerns, precludes the commercial viability of an immunoassay incorporating mass-produced native protein(s) as the antibody capture antigen(s). In so being, to produce a robust serological assay to detect specific, and possibly conformationally sensitive, antibodies, a source of high-yield, highly pure, and correctly folded recombinant antigen(s) needs to be identified and evaluated (6). With each of these considerations in mind, we synthesized a recombinant form of an *M. tuberculosis* CF protein in the yeast *P. pastoris* and then evaluated its immunogenicity and serodiagnostic potential compared to the same protein expressed in *E. coli*.

CF proteins are secreted or released by growing *M. tuberculosis* into the culture medium. One characteristic of CF proteins as a whole, and of many individual CF proteins, is their strong immunostimulatory capacity (4, 9, 31). Indeed, the production of CF proteins is believed to account for the heightened efficacy of live, as opposed to killed, *M. tuberculosis* vaccines in animal models (1, 12). Containing more than 200 different proteins, the CF presents an abundance of candidate antigens for use in developing a viable serodiagnostic immunoassay for TB (32). One such *M. tuberculosis* CF protein is CFP32, a 32-kDa putative bimodular glyoxalase of unknown function. CFP32 has been shown to be expressed in the lungs of TB patients and is known to stimulate a humoral antibody response (13, 26). The fact that all *M. tuberculosis* strains evaluated to date (>600) by PCR or Southern blot for *cfp32* have been found to be in possession of the gene indicates that CFP32 serves a necessary biological role (13, 14; R. C. Huard, H.-X. Zhu, K. Menon, S. Chitale, R. Senaratne, D. Fathallah, C. Benabdesselem, M. R. Barbouche, J. Belisle, L. Riley, and J. L. Ho, unpublished data). Importantly, unlike other CF proteins that have been evaluated as serodiagnostic antigens, such as the antigen 85 complex proteins (15), CFP32 is restricted to members of the MTC and has not been identified in environmental mycobacteria (13, 14). Recently, CFP32 was recognized as the enzymatic mediator of *M. tuberculosis*-specific neutral red dye cytochemical staining, a classical test once used to differentiate virulent *M. tuberculosis* from nontuberculous mycobacteria (3). Therefore, in being a conserved MTC-restricted antigenic CF protein, we believed CFP32 to be a prime antigen for use in a specific serodiagnostic test for TB.

The results of our investigation indicate that antigenic epitopes were more readily available for antibody recognition in the yeast-produced rCFP32 as opposed to the rCFP32 generated in *E. coli* by both Western blot studies as well as in a comparative ELISA. In further testing, the ELISA incorporating yeast rCFP32 as the antibody capture antigen was able to discriminate TB case patients from *M. tuberculosis*-unexposed–BCG-naive healthy controls and healthy BCG vaccinees with a high sensitivity (100% and 85%) and high specificity (100% and 98%), respectively. Notably, this de novo assay showed a significant improvement over a previously evaluated ELISA that incorporated *E. coli* rCFP32 as the screening antigen (13) and also performed better than the parallel *E. coli* rCFP32-based immunoassay to which it was compared in the present study. Moreover, not only was the serologic response of TB patients and BCG-vaccinated persons significantly greater for yeast rCFP32 than *E. coli* rCFP32 but the differential mean reactivity between these groups was greater in the case of rCFP32 produced in yeast, elevating the usefulness and appeal of this antigen for use as a serodiagnostic marker. Because CFP32 is expressed by all MTC subspecies, including BCG (13), it was not unexpected that residual antibodies were present in individuals vaccinated with BCG over two decades previous. In using the serological responses of BCG-vaccinated persons to set a cutoff for immune reactivity to CFP32, com-

pared to the PPD-negative–BCG-naive cohort, the sensitivity of the yeast rCFP32-based assay dropped by 15%. However, given that BCG remains one of the most widely used vaccines, the BCG-vaccinated cohort arguably represents a more relevant control than the PPD-negative–BCG-naive group in a TB serodiagnosic test applied in certain contexts, such as Tunisia, with the important point being that, even in this situation, the yeast rCFP32-based assay remained highly sensitive and specific. It should also be acknowledged that we did not evaluate a cohort comprised of healthy PPD-positive, presumably *M. tuberculosis*-exposed, persons. Such a cohort of latently infected persons will be included in a wider evaluation currently in the planning stages. At present, we do not know if the yeast rCFP32 will be able to segregate latent TB from active disease. We hope that the assay will allow us to predict which persons are likely to reactivate latent TB or are manifesting early active disease. In any case, based upon the present data, the rCFP32 produced in *P. pastoris* seems to have good potential for the serological diagnosis of TB. It is further worth mentioning that, in a separate study, and unlike the *E. coli* version of rCFP32, the yeast-generated rCFP32 exhibited functional properties similar to those of native CFP32 (Huard et al., unpublished). This point underscores the need to produce recombinant protein in systems that approximate the native antigen.

In most reports, assays based upon *E. coli*-expressed recombinant *M. tuberculosis* proteins appear to have significantly lower sensitivities compared to the native counterpart antigen, and this has been linked to differences in glycosylation patterns (11, 23). Since B-cell epitopes are known to recognize specific conformational structures, species-restricted differences in posttranslational modifications may translate to a reduced number of antigenic epitopes for antibody binding (11, 23). We hypothesize that the surprisingly high sensitivity of the single-antigen TB serodiagnostic test using yeast rCFP32, compared to that using the same *E. coli* expressed antigen, obtained in the current Tunisian cohort is probably due to differences in such posttranslational events. The presence of shared forms of protein posttranslational modifications between *P. pastoris* and *M. tuberculosis* remains to be fully characterized. Interestingly, recent data (34) support the possibility that certain pathways and forms of protein posttranslational modification in eukaryotes are evolutionarily conserved in *M. tuberculosis* and, specifically, that these similarities are shared by yeasts, thereby justifying our choice of *P. pastoris* as a surrogate host for the production of rCFP32. Weldingh et al. (36) individually screened the seroreactivity of patient sera to 35 single recombinant *M. tuberculosis* proteins expressed in *E. coli*, including CFP32 (given as TB27.3). Although their rCFP32 exhibited a degree of seroreactivity in their selected TB cohort, compared to controls, it was not further analyzed because their minimum serological response threshold ratio was not met for this protein. Our data suggest that their failure to mark CFP32 (and other known immunologically active *M. tuberculosis* antigens such as ESAT-6) may have been because they produced their recombinant antigens in *E. coli*. Our data highlight that the mode of antigen production can be a factor limiting the identification of seroreactive antigens. Indeed, for this reason, many potential serodiagnostic antigens for TB or other diseases may have been overlooked in prior studies because their immunogenicity was underestimated, and these should be reproduced using alternate surrogate hosts and reevaluated.

It is generally accepted that the most likely format of a truly robust serological assay for TB will incorporate a combination of several different *M. tuberculosis* antigens, including proteins such as ESAT-6 and CFP10 that are absent in BCG, thereby allowing a necessary differentiation of *M. tuberculosis*-infected persons from BCG vaccinees. To our knowledge, the sensitivity of a serologic test for TB using a single antigen has rarely exceeded 80% (17, 36). For this reason, the high sensitivity and specificity of our single-antigen assay was unexpected. Our data, therefore, raise the hope that a high-performance TB serodiagnostic test will eventually be developed and may require just a minimum set of antigens, depending upon their method of manufacture.

In the current study, the results of serological testing from two persons were excluded from analysis but raised some intriguing possibilities. One individual in the healthy BCG-vaccinated cohort had a strong TST result (suggestive of exposure to *M. tuberculosis*) and high serological response to yeast rCFP32. These data indicate that the assay developed to detect anti-CFP32 antibodies may be useful in identifying cases of recent *M. tuberculosis* infection. The limitations of the TST were recently summarized by Pai et al. (19). Therefore, a multivalent serological assay incorporating yeast rCFP32 may overcome these restrictions and serve as a confirmation or replacement of PPD screening. As related to the other regularly BCG-vaccinated persons and the BCG-naive infants, the combined data support the possibility that BCG expresses immunogenic CFP32 in vivo but, as indicated by the data from the other excluded individual who was BCG vaccinated for a third time and had a good serological response to yeast rCFP32, this response wanes over time. BCG is known to protect against certain forms of childhood TB (2). Could this potential decline in anti-CFP32 humoral responses also exist for other BCG antigens and underlie, in part, the inconsistent protection of BCG vaccination from adult forms of TB? There is a growing interest in BCG boosting of adults, so with respect to the thrice-BCG-vaccinated individual, the data suggest that perhaps a serological assay such as ours could prove useful when monitoring the immunological response to BCG revaccination and provide a correlate of protective immunity. Of course, the above speculations are based on single subjects, and the questions raised can only be answered by further research. Nonetheless, our data provide strong evidence that the expression of *M. tuberculosis* proteins in *P. pastoris* may be a better modality for producing antigens of diagnostic potential for both TB and other infectious diseases, representing an advance with potentially far-reaching applications that merits further investigation.

In conclusion, our study demonstrates that *P. pastoris*-produced rCFP32 displayed enhanced antibody binding compared to *E. coli* rCFP32, both by laboratory-raised antibodies and by sera from TB patients or persons vaccinated with BCG. Testing with yeast rCFP32 resulted in an 85% sensitivity in diagnosing TB patients and a specificity of 98% when using healthy BCG-vaccinated persons to establish a cutoff. Our data provide a sound basis for a larger-scale comparison between rCFP32 produced by *P. pastoris*, *E. coli*, and mycobacteria as serologic test antigens to discriminate active TB from latent *M. tubercu-*

losis infection or prior BCG vaccination. Together with CFP32, a panel of *M. tuberculosis* antigens expressed in yeast may provide a rapid and low-cost approach for diagnosing TB with a sensitivity that is at least comparable to that of mycobacterial culture.

ACKNOWLEDGMENTS

We are grateful to H. Mardassi for providing banked sera from TB patients and A. Rebai for help in carrying out the preliminary statistical analysis. We also thank Warren D. Johnson, Jr., for the support needed to complete this project.

This work was funded by the Tunisian Ministry for Scientific and Technological Research, with a contribution from the NIH NIAID grants R21 AI062332 (J.L.H.), FIC 3 D43 TW000018, and WHO A50500.

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